Effect of airway metagenomic and immunological changes on pulmonary function tests in smokers and smokeless tobacco users

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SRI BALAJI VIDYAPEETH

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by

Mr. MANIKANDAN S

(UIN: 1805001003)

Under the guidance of

Dr. NIKHILESH SINGH
Professor, Department of Physiology
Mahatma Gandhi Medical College & Research Institute
Sri Balaji Vidyapeeth

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1. ABSTRACT

Background

Tobacco consumption in both smoking and smokeless forms is a major public health concern and a well-established risk factor for respiratory morbidity. Emerging evidence suggests that tobacco exposure not only impairs pulmonary function and induces systemic inflammation but also alters the airway microbiome, potentially contributing to early lung disease. However, comparative data on the effects of smoking and smokeless tobacco use on airway metagenomics, immunological changes, and pulmonary function remain limited.

Methods

This analytical cross-sectional study included 90 male participants aged 25–45 years, comprising 30 smokers, 30 smokeless tobacco users, and 30 healthy controls. Pulmonary function tests were performed using spirometry and systemic inflammatory markers. The airway microbiome was characterized using 16S rRNA gene sequencing, following which targeted molecular sequencing was performed for selected respiratory pathogens identified based on the microbiome profiling results. Statistical analysis was performed using appropriate parametric and non-parametric tests for group comparisons, along with multivariate and microbiome-specific analytical approaches.

Results

Predicted pulmonary function values were significantly reduced across all parameters in both tobacco-using groups. Smokers exhibited significantly reduced FEV_1, FEV_1/FVC ratio, PEFR, and $FEF_{25–75\%}$, while smokeless tobacco users showed reduced PEFR and $FEF_{25–75\%}$ compared with controls. Smokers demonstrated elevated neutrophil percentage, neutrophil–lymphocyte ratio (NLR), and hs-CRP levels, whereas smokeless tobacco users showed increased eosinophil percentage and hs-CRP.

Microbiome profiling revealed no significant differences in within-group alpha diversity or between-group beta diversity; however, differential abundance analysis identified enrichment or depletion of specific bacterial genera in tobacco-using groups. Forward constrained ordination analysis did not demonstrate a significant association between pulmonary microbial community structure and the studied pulmonary function or inflammatory parameters. Conversely, reverse constrained ordination analysis showed significant associations of white blood cell count and hs-CRP with pulmonary microbial community

structure in group-wise comparisons, of which only NLR remained significant after adjustment for tobacco exposure. Multivariable association analysis similarly did not identify any bacterial taxa showing a significant independent association with these clinical variables. Mediation analysis suggested that these associations primarily reflected a direct effect of tobacco exposure on airway microbiome composition rather than mediation through systemic inflammation.

Based on microbiome profiling results, targeted molecular sequencing was performed for selected respiratory pathogens. PCR analysis of sputum samples demonstrated increased detection of *Streptococcus pneumoniae* and *Haemophilus influenzae* in both smokers and smokeless tobacco users, with *H. influenzae* showing an independent negative association with FVC, while tobacco exposure was independently associated with reduced FEV_1 and PEFR and increased inflammatory markers

Conclusion

In conclusion, both smoking and smokeless tobacco use are associated with impaired pulmonary function and elevated systemic inflammation, with smokers showing broader deficits and higher neutrophil-linked inflammation compared with smokeless tobacco users, who show eosinophil-linked inflammation. The respiratory microbiome shows shifts in specific bacterial genera that differ between the groups but does not independently influence clinical parameters, with tobacco exposure driving the observed associations. Tobacco use also increases detection of key respiratory pathogens, highlighting early airway microbial alterations.

Keywords: Pulmonary function test, Smoker, Smokeless tobacco users, 16S rRNA gene sequencing, Streptococcus pneumoniae, Haemophilus influenzae

2. INTRODUCTION

Tobacco use remains a major global public health challenge. Globally, approximately 1.3 billion people consume tobacco, contributing to nearly 7 million deaths annually. [1] In India, around 330 million individuals use tobacco, accounting for more than one million deaths each year. Tobacco is commonly consumed in both smoked forms, such as bidi and cigarettes, and smokeless forms, including khaini, gutkha, Hans, betel quid, and zarda.[2] Tobacco consumption is a well-established risk factor for several chronic respiratory diseases, including chronic obstructive pulmonary disease (COPD), pneumonia, and lung cancer, which rank among the leading causes of global morbidity and mortality. Tobacco smoke contains more than 7,000 chemical compounds, of which at least 69 are known carcinogens, while smokeless tobacco products contain approximately 4,000 chemicals, including nearly 28 carcinogenic substances. These toxic constituents impair ciliary motility and mucociliary clearance by damaging the airway epithelium and altering mucus properties, leading to reduced lung function and increased susceptibility to respiratory infections. Pulmonary function test parameters serve as sensitive tools for identifying early airway dysfunction and subclinical lung impairment. [3] Chronic exposure to tobacco results in persistent airway epithelial injury and activation of alveolar macrophages, triggering the release of pro-inflammatory cytokines such as interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor-α (TNF-α). These mediators drive both local airway inflammation and systemic inflammatory responses, reflected by elevated white blood cell (WBC) count, neutrophil-to-lymphocyte ratio (NLR), and C-reactive protein (CRP) levels.[4] Prolonged inflammation and continuous tobacco exposure impair innate immune cell function and suppress the production of key immune molecules, including cytokines, antimicrobial peptides, and antibodies, thereby facilitating persistent bacterial colonization and increasing vulnerability to respiratory infections.[5] Recent advances in culture-independent molecular techniques, particularly 16S rRNA gene amplicon sequencing, have highlighted the importance of the airway microbiome in maintaining respiratory health. Alterations in airway microbial communities have been implicated in immune dysregulation, chronic inflammation, and progressive decline in pulmonary function across various respiratory diseases. [6] Tobacco exposure can modify the airway microenvironment by altering oxygen tension, pH, nutrient availability, and immune surveillance, thereby promoting microbial dysbiosis and pathogenic bacterial overgrowth. While smoking directly exposes the airway epithelium to toxicants, smokeless tobacco may influence lung health indirectly through systemic inflammation and immune modulation.

However, it remains unclear whether bacterial colonization influences immunological and inflammatory markers and pulmonary function when comparing smokers and smokeless tobacco users. To address this gap, the present study aims to investigate the effects of airway metagenomic alterations and immunological changes on pulmonary function among smokers and smokeless tobacco users, to identify potential microbial and inflammatory markers for the early detection and prevention of tobacco-related lung diseases.

3. AIM AND OBJECTIVES

AIM: To investigate the effects of airway metagenomic and immunological changes on pulmonary function among tobacco users and identify potential bacteria for early detection and prevention of tobacco-related lung diseases.

Objectives:

1. To compare the pulmonary function test parameters among smokers, smokeless tobacco users, and controls.

2. To assess immunological and inflammatory changes by using complete blood count (CBC) and high-sensitivity C-reactive protein (hs-CRP) among the groups

3. To analyze the associations between the airway sputum microbiome (profiled using 16S rRNA gene amplicon sequencing), immunological changes, and pulmonary function among the study groups.

4. To identify potential bacterial species in sputum samples by using targeted molecular sequencing.

4. RESEARCH QUESTION

Does the potential bacterial colonization alter the immunological and pulmonary function test among smoking and smokeless tobacco users when compared to the controls?

5. BRIEF REVIEW OF LITERATURE

Cigarette smoke contains a complex mixture of toxic compounds, including formaldehyde, phenol, acrolein, and carbon monoxide, which exert deleterious effects on the respiratory epithelium. These constituents impair mucociliary clearance by damaging ciliated epithelial cells and altering airway mucus, thereby compromising one of the primary innate defense mechanisms of the respiratory tract (Yadav et al., 2014). Structural damage to the ciliated epithelium leads to mucus hypersecretion and delayed clearance, creating a favorable microenvironment for microbial persistence and colonization within the airways (Feldman et al., 2013). In addition to mechanical disruption, cigarette smoke generates excessive amounts of reactive oxygen species (ROS) and reactive nitrogen species (RNS), including superoxide anions, hydrogen peroxide, hydroxyl radicals, and peroxynitrite. These reactive molecules induce oxidative stress and cytotoxic injury to alveolar epithelial cells, resulting in impaired epithelial integrity and increased susceptibility to infection (Aoshiba et al., 2003). Oxidative

stress further activates redox-sensitive inflammatory pathways, such as NF-κB and MAPK signaling, thereby sustaining chronic airway inflammation and promoting airway remodeling.

Unlike smoking, smokeless tobacco exerts its effects primarily through systemic absorption of nicotine, tobacco-specific nitrosamines, and heavy metals, which induce widespread oxidative stress and systemic inflammation. These systemic effects promote airway remodeling, epithelial thickening, and recruitment of inflammatory cells to the respiratory mucosa, ultimately leading to impaired pulmonary function. Although the route of exposure differs from smoking, smokeless tobacco use similarly contributes to airway inflammation and structural alterations that compromise respiratory function (Shindhe et al., 2018).

Tobacco-induced immune dysfunction plays a critical role in facilitating bacterial colonization of the airways. Exposure to tobacco products impairs the phagocytic function and bactericidal activity of alveolar macrophages and neutrophils, while simultaneously enhancing microbial virulence under tobacco-exposed conditions. Furthermore, smoking suppresses adaptive immune responses by reducing T- and B-lymphocyte proliferation, thereby weakening host defense mechanisms against respiratory pathogens (Bagaitkar et al., 2008). Tobacco exposure also increases bacterial adhesion to epithelial cells and disrupts mucociliary clearance, collectively favoring persistent colonization by potentially pathogenic microorganisms in the respiratory tract (Charlson et al., 2010).

In healthy individuals, the composition of the lung microbiome is maintained through a dynamic balance between microbial immigration and microbial elimination, with minimal contribution from local microbial replication (Yi X et al., 2022). Smoking profoundly disrupts this equilibrium by altering the airway microenvironment, including changes in pH, oxygen tension, nutrient availability, temperature, and inflammatory status. Reduced oxygen availability within the airways favours the growth of anaerobic pathogenic bacteria while concurrently suppressing beneficial microbial taxa. These ecological changes lead to a shift in the lung microbiome, which has been implicated in chronic inflammation, immune dysregulation, and progressive decline in pulmonary function (Huang et al., 2019).

Collectively, existing evidence suggests that tobacco use causes epithelial injury, oxidative stress, immune dysfunction, and changes in the airway microbiome, leading to a sustained cycle of inflammation, microbial imbalance, and lung function impairment. However, studies that comprehensively assess airway microbiome changes, systemic inflammation, and pulmonary function in smokers and smokeless tobacco users remain limited.

6. MATERIALS AND METHODS

Study Design: Analytical cross-sectional study

Sampling technique: Purposive sampling

Study population: Smoking tobacco users, smokeless tobacco users and controls

Study Area: Puducherry, Cuddalore, Neyveli, Chidambaram, Kattumannarkoil.

Sample collection period: April 2023 to February 2025

Sample Size:

The sample size was calculated based on the comparison of two independent means using a two-sample mean difference approach. An alpha error of 5% and a beta error of 20% (power of 80%) were considered. Based on previous literature reported by Kim HJ et al. (2017), the mean values for Group 1 and Group 2 were 103.5 ± 12.4 and 92.8 ± 16.2, respectively, with a 1:1 group allocation ratio. The calculated minimum sample size required was 30 participants per group.

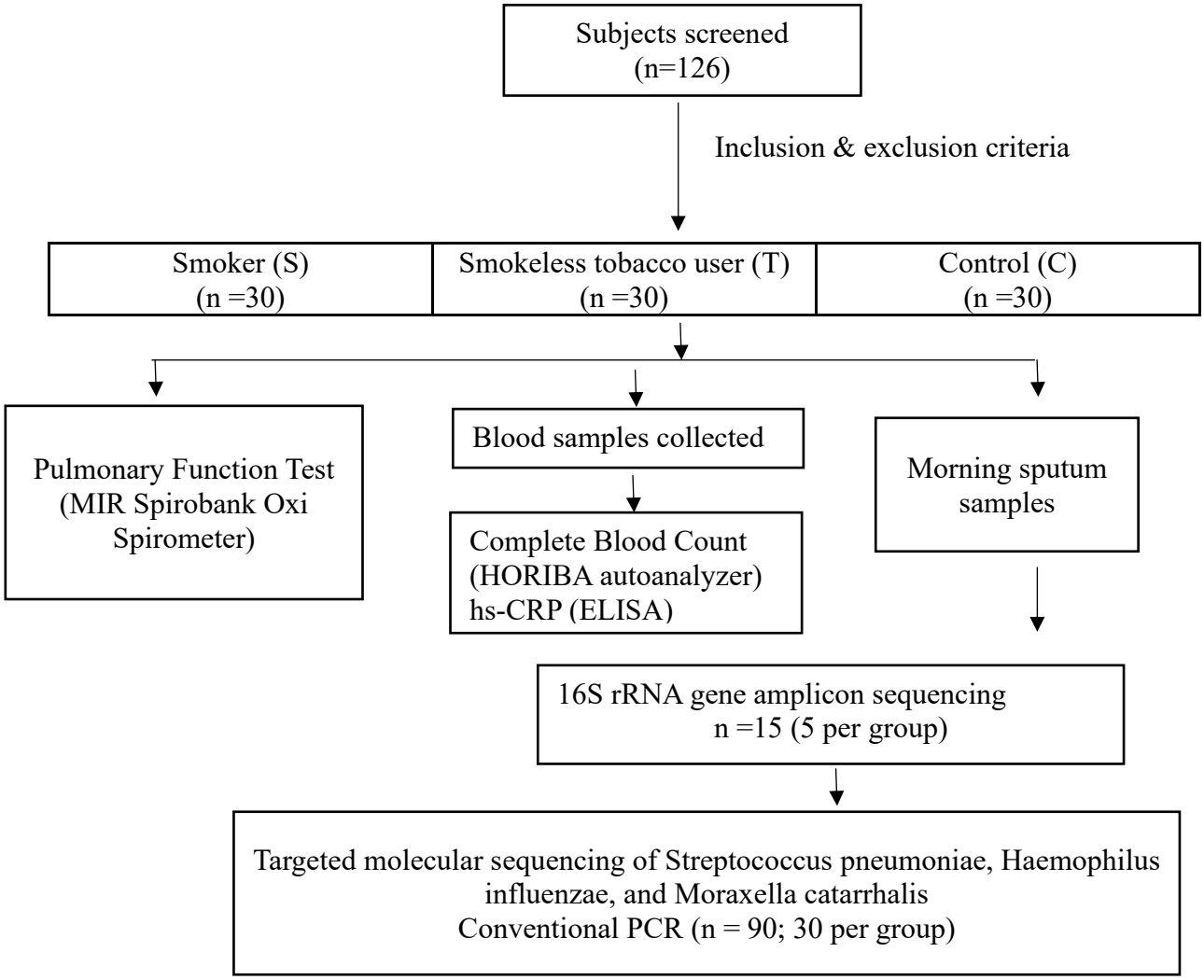
Inclusion criteria

- Smokers - A person who smokes any tobacco product, either daily or occasionally, at the time of the survey or assessment. (WHO, 2018)
- Smokeless tobacco user - use of any smokeless tobacco product either daily or occasionally, at the time of the survey. (WHO, 2018)
- Non-tobacco users - never used smoke & smokeless tobacco products
- Male & 25 to 45 years
- Willing to participate in all the tests.

Exclusion criteria

- The person with an occupational history of working in textile mills and cement factories, coal factories, or other places where lungs are affected by dust or fumes
- History of respiratory diseases (asthma, COPD, bronchitis, pneumonia, tuberculosis, COVID-19)
- Any other inflammatory or infectious conditions (e.g., meningitis, sinusitis)
- Undergoing any immunosuppressive treatments

Methodology



7. STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS software (version 21). Data normality was assessed using the Shapiro–Wilk test. Normally distributed variables were analyzed using one-way analysis of variance (ANOVA) and are presented as mean ± standard deviation (SD), while non-normally distributed variables were analyzed using the Kruskal–Wallis test and are expressed as median with interquartile range (IQR). Mann–Whitney U test was used for subgroup analysis of non-parametric data. Multiple linear regression was performed to identify independent predictors and assess associations between the variables.

Microbiome analysis was performed using RStudio (version 2025). Microbial community diversity within samples (α-diversity) was assessed using richness, the Shannon index, and the Simpson index, while differences in community composition between groups (β-diversity) were evaluated using non-metric multidimensional scaling (NMDS) for visualization, permutational multivariate analysis of variance (PERMANOVA), and beta dispersion. Differential abundance analysis was performed using relative abundance and DESeq2 to identify significantly different bacterial genera. Associations between microbial genera and clinical variables were examined using distance-based redundancy analysis (db-RDA) and multivariable association with linear models (MaAsLin2), and mediation analysis was conducted to assess the indirect and direct effects of inflammatory markers on the relationship between tobacco exposure and microbiome alterations.

8. Results

There was no statistically significant difference in baseline physical parameters among the control, smoker, and smokeless tobacco user (Table 1).

Table1: Comparison of baseline physical parameters among the three groups

Physical parameters (n = 30)	Control (Mean ± SD)	Smoker (Mean ± SD)	Smokeless tobacco user (Mean ± SD)	F- value	P.value
Age (years)	39.1± 8.2	40.6±9.2	38.3 ± 7.1	0.713	0.492
Height (cm)	165.1+7.6	167± 9.1	169.6± 6.9	2.67	0.073
Weight (kg)	68.3 ± 13.6	69.6±12.8	69.8 ± 13.9	0.124	0.883
BMI (kg/m²)	24.9± 3.5	25.1± 3.9	24.3 ± 4.8	0.339	0.713

One–Way ANOVA test

All pulmonary function parameters (predicted % values), FVC%, FEV_1%, FEV_1/FVC%, PEFR%, and $FEF_{25–75}$% were significantly reduced in both smokers and smokeless tobacco users compared with controls (Table 2).

Table 2: Comparison of the pulmonary function test among three groups (predicted percentage)

PFT Parameters (n=30)	Control (Mean ± SD)	Smoker (Mean ± SD)	Smokeless tobacco user (Mean ± SD)	F- value	P-value
FVC (%)	90.0±7.6	82.3± 8.6 [a]	83.7±8.9[a]	7.2	0.001*
FEV_1 (%)	88.0± 9.3	84.1±8.7[a]	81.5±8.8[a]	3.9	0.02*
FEV_1/FVC (%)	99.5±6.1	88.1±9.6[a]	88.9±9.4[a]	16.8	0.001**
PEFR (%)	96.2± 15.0	83.8± 13.5[a]	87.8 ± 12.4[a]	6.4	0.003**
FEF_{25-75} (%)	88.3±10.2	71.8±10.7[a]	69.2±15.3[a]	21.3	0.001**

One–way ANOVA - Post hoc Tukey HSD . a – significantly different from when compared to the control.

Absolute pulmonary function values showed that smokers had significantly lower FEV_1, FEV_1/FVC ratio, PEFR, and $FEF_{25–75\%}$ compared with the control group. Smokeless tobacco users also exhibited significantly lower PEFR and $FEF_{25–75\%}$ compared with the control group (Table 3).

Table 3: Comparison of pulmonary function test among three groups - absolute value (L)

PFT Parameters (n=30)	Control (Mean ± SD)	Smoker (Mean ± SD)	Smokeless tobacco user (Mean ± SD)	F- value	P-value
FVC (L)	3.5 ± 0.5	3.3 ±0.4	3.2±0.6	1.9	0.14
FEV_1 (L)	3.0± 0.3	2.7±0.4[a]	3.0 ± 0.4	5.9	0.004*
FEV_1/FVC (%)	82.4± 2.5	79.6 ± 4.5 [a]	80.2 ± 5.4	3.4	0.03*
PEFR(L/min)	512.5 ± 42.3	451.5 ± 77.8[a]	474 ± 72.0[a]	6.5	0.002**
$FEF_{25-75\%}$(L/s)	3.4 ± 0.7	2.9± 0.8[a]	2.9 ± 0.9[a]	3.6	0.03*

One–way ANOVA -Post hoc Tukey HSD & Tamhane. a – significantly different when compared to the control.

Smokers exhibited significantly higher NLR and hs-CRP levels compared with controls, whereas smokeless tobacco users showed significantly increased eosinophil (%) compared with both controls and smokers. In addition, hs-CRP levels were significantly elevated in smokeless tobacco users compared with controls (Table 4a). Smokers showed a significantly higher neutrophil (%) compared with controls (Table 4b).

Table 4a: Comparison of blood parameters among the three groups

Blood Parameters (n=30)	Control (Median, IQR)	Smoker (Median, IQR)	Smokeless tobacco user (Median, IQR)	KW value	P.Value
WBC (cells /mm^3)	8100 (6750-9400)	7600 (6575-8525)	7000 (5825-7967)	4.48	0.11
Eosinophils (%)	3(1.4,50)	2.8(2.0,3.7)	6.9 (2.6,11.5) [ab]	11.0	0.004*
Basophils (%)	1.0(0.4,1.0)	0.8 (0.6,1.0)	0.5 (0.1,0.8)	6.2	0.05
Monocytes (%)	8.0(6.3,8.7)	7.4(3.0,9.4)	7.0 (6.0,8.3)	0.85	0.65
NLR	1.4(1.2,1.6)	2.0 (1.4,2.0) [a]	1.4 (1.3,1.8)	6.7	0.03*
hs-CRP(mg/dl)	0.8(0.4,1.0)	3.1 (1.6,4.4) [a]	3.1(2.8,3.8) [a]	36.3	0.001*

Kruskal–Wallis test - Dunn's post hoc test. a – significantly different from when compared to the control, b – significantly different when compared to the smoker

Table 4b: Comparison of blood parameters among the three groups

Blood parameters (n=30)	Control (Mean ± SD)	Smoker (Mean ± SD)	Smokeless tobacco user (Mean ± SD)	F.value	P.value
Neutrophils %	50.1 ± 6.4	54.8 ±8.2[a]	53.1 ± 5.8	3.4	0.03*
Lymphocytes %	34.5 ± 6.6	33.2 ±7.4	31.5 ± 6.9	1.8	0.16

One-way Anova - Post hoc Tukey HSD. a – significantly different when compared to the control

No significant differences were observed in alpha diversity (Table 5).

Table 5: Comparisons of alpha diversity indices among the three groups

Alpha diversity Index	χ^2	p-value
Shannon	4.88	0.08
Richness	3.42	0.18
Simpson	2.24	0.32

Beta diversity analysis revealed no statistically significant differences among the study groups (Table 6a).

Table 6a: Comparison of beta diversity among groups using PERMANOVA

	R^2	F – value	P.value	p.adj
Overall - Df (2) sum of Sqs 0.50	**0.19**	1.47	0.134	-

Permutational multivariate analysis of variance (PERMANOVA)

Beta dispersion analysis showed no significant differences among the study groups, indicating similar within-group variability in microbial community composition (Table 6b).

Table 6b: Comparison of Beta Dispersion Across Groups

Beta dispersion	Df	X^2	P.Value	P-adj
Overall (distance to centroid)	2	0.26	0.87	----

Taxa-level analysis based on relative abundance showed that the dominant bacterial genera across all three groups were *Streptococcus*, *Escherichia*, *Prevotella*, *Neisseria*, *Veillonella*, *Pseudomonas*, *Leptotrichia*, *Staphylococcus*, and *Haemophilus* (Table 7a)

Table 7a: Taxa-level analysis of the top 10 dominant genera across all samples

Genus	Relative abundance (%)
Streptococcus	19.8
Escherichia	16.8
Prevotella	11.1
Neisseria	8.6
Veillonella	7.2
Pseudomonas	5.7
Leptotrichia	5.5
Staphylococcus	4.9
Haemophilus	4.5
Unclassified	15.9

Differential abundance analysis showed 107 significantly different bacterial genera between controls and smokers, 17 significantly different genera between controls and smokeless tobacco users, and 51 significantly different bacterial genera between smokers and smokeless tobacco users (Table 7b).

Table 7b: Differential abundance analysis between study groups (DESeq2)

Group comparison	Number of significant bacterial genera
Control vs Smoker	107
Control vs Smokeless tobacco user	17
Smoker vs Smokeless tobacco user	51

DESeq -2 Differential Expression using Sequencing – version 2

Table 7c: Top 20 Enriched Bacterial Genera Between Groups (DESeq2)

Enriched in Smoker	Enriched in Control
Leptotrichia_A *Haemophilus_D* *Prevotella* *Veillonella_A* *Porphyromonas_A* *Streptococcus* *Gemella* *Neisseria* *Granulicatella* *Pseudomonas_E*	*Escherichia* *Pauljensenia* *Unclassified* *Staphylococcus* *Acinetobacter* *SIO2CI* *Kluyvera* *Ligilactobacillus*

Enriched in Smokeless tobacco user	Enriched in control
Escherichia *Pseudomonas* *Unclassified* *Porphyromonas* *Actinetobacter* *Streptococcus* *Veillonella* *Haemophilus D* *Granulicatella*	*Kluyvera* *Ligilactobacillus* *SIO2C1* *Prevotella* *Gemella* *Pauljensenia* *Leptotrichia* *Rothia* *Neisseria* *Haemophilus A*

Enriched in Smokeless tobacco user	Enriched in Smoker
Staphylococcus *Escherichia* *Ligilactobacillus* *Kluyvera* *SIO2C1* *Acinetobacter* *Unclassified* *Pseudomonas*	*Granulicatella* *Pauljensenia* *Porphyromonas* *Streptococcus* *Veillonella* *Gemella* *Haemophilus D* *Rothia* *Prevotella* *Neisseria* *Leptotrichia* *Haemophilus A*

No significant association between microbial community composition and the assessed clinical parameters (Table 8).

Table 8: Association of microbiome with clinical variables

Clinical variables	p. partial	q. partial
FVC (L)	0.62	0.86
FEV_1 (L)	0.76	0.86
FEV_1/FVC%	0.83	0.86
PEFR (L/min)	0.6	0.86
$FER_{25-75\%}$ (L/s)	0.91	0.91
WBC (cells/mm^3)	0.057	0.35
Neutrophil (%)	0.22	0.65
Lymphocyte (%)	0.23	0.65
Eosinophil (%)	0.72	0.86
Basophil (%)	0.41	0.79
Monocyte (%)	0.17	0.65
NLR	0.054	0.35
hs- CRP (mg/dl)	0.23	0.65

Forward constrained ordination: Distance-based Redundancy Analysis (db-RDA)

Pairwise db-RDA analysis demonstrated no significant associations between significant bacterial genera and clinical parameters (Table 9).

Table 9: Pairwise db-RDA analysis of significant bacterial genera associated with clinical parameters

Clinical Variables	C vs S (107)		C Vs T (17)		S vs T (51)	
	p.P	q.P	p.P	q.P	p.P	q.P
FVC (L)	0.54	0.86	0.90	0.98	0.62	0.83
FEV_1 (L)	0.74	0.88	0.13	0.66	0.65	0.83
FEV_1/FVC%	0.85	0.90	0.95	0.99	0.80	0.83
PEFR (L/min)	0.63	0.86	0.81	0.98	0.59	0.83
$FER_{25-75\%}$ (L/s)	0.67	0.86	0.45	0.83	0.76	0.83
WBC (cells/mm^3)	0.03	0.40	0.08	0.65	0.10	0.42
Neutrophil (%)	0.30	0.73	0.30	0.83	0.10	0.42
Lymphocyte (%)	0.21	0.66	0.55	0.91	0.09	0.42
Eosinophil (%)	0.65	0.86	0.78	0.98	0.81	0.83
Basophil (%)	0.24	0.66	0.45	0.83	0.37	0.76
Monocyte (%)	0.08	0.41	0.16	0.66	0.17	0.54
NLR %	0.07	0.41	0.47	0.83	0.02	0.41
hs- CRP (mg/dl)	0.35	0.73	0.16	0.66	0.25	0.59

Forward constrained ordination: Distance-based Redundancy Analysis (db-RDA)
p. Partial, q. Partial

Reverse db-RDA analysis was performed to find the reverse interaction between the airway microbiome and clinical parameters. It revealed a significant association between WBC count and the global respiratory microbiome in smokers (Table 10).

Table 10: Effect of inflammatory markers on bacterial genera

Clinical parameters	C	S	T
	p. value		
WBC (cells/mm^3)	0.57	0.01*	0.12
Neutrophil (%)	0.48	0.98	0.65
Lymphocyte (%)	0.43	0.58	0.30
Eosinophil (%)	0.70	0.38	0.19
Basophil (%)	0.80	0.67	0.85
Monocyte (%)	0.47	0.44	0.40
NLR	0.35	0.93	0.33
hsCRP (mg/dl)	0.33	0.23	0.20

Reverse constrained ordination - Distance-based Redundancy Analysis (db-RDA)

Pairwise reverse db-RDA analysis indicated that WBC count was associated with significant bacterial genera across all pairwise comparisons (controls vs smokers, controls vs smokeless tobacco users, and smokers vs smokeless tobacco users). In addition, hs-CRP showed an association with significant bacterial genera in the comparison between controls and smokeless tobacco users. A trend toward association was also observed for NLR in the control vs smokeless tobacco user and smoker vs smokeless tobacco user comparisons (Table 11).

Table 11: Pairwise relationship of clinical parameters on significantly different bacterial genera

Clinical parameters	C vs S p.value	C vs T p.value	S vs T p.value
WBC (cells/mm^3)	0.003*	0.005*	0.002*
Neutrophil (%)	0.223	0.268	0.194
Lymphocyte (%)	0.267	0.311	0.237
Eosinophil (%)	0.236	0.216	0.216
Basophil (%)	0.317	0.3	0.308
Monocyte (%)	0.283	0.311	0.27
NLR%	0.294	0.056	0.083
hsCRP (mg/dl)	0.056	0.001*	0.113

Reverse constrained ordination - Distance-based Redundancy Analysis (db-RDA)

After adjustment for tobacco exposure, only the neutrophil-to-lymphocyte ratio (NLR) remained significantly associated with the microbiome (Table 12).

Table 12: Partial db-RDA of clinical parameters controlling for tobacco

Clinical parameters	P.value	FDR
WBC (cells/cumm)	0.69	0.9
Neutrophil (%)	0.1	0.85
Lymphocyte (%)	0.2	0.85
Eosinophil (%)	0.72	0.9
Basophil (%)	0.38	0.9
Monocyte (%)	0.71	0.9
NLR	0.04*	0.85
hs- CRP (mg/dl)	0.2	0.85

Partial db-RDA - Distance-based Redundancy Analysis

Mediation analysis was performed because the neutrophil-to-lymphocyte ratio (NLR) remained associated with the microbiome after adjustment for tobacco exposure and also showed a trend toward association in comparisons between smokers and smokeless tobacco users and between controls and smokeless tobacco users. No mediating effect of NLR was observed in the smoker versus smokeless tobacco user comparison, indicating a predominant role of direct tobacco exposure (Table 13a).

Table 13a: Mediation analysis of the Smoker vs smokeless tobacco user effect on significant genera via NLR

Effect	Estimate	Confidence Interval (95%)	p-value
ACME (Indirect Effect)	-4.91	(-15.58 - 4.88)	0.282
ADE (Direct Effect)	30.8	(17.59 - 41.36)	0.002 **
Total Effect	25.9	(11.53 - 38.07)	0.0001 ***
Proportion Mediated	-0.189	(-0.97 - 0.170)	NA

Exposure (X): Tobacco group (S vs T), Mediator (M): NLR, Outcome (Y): significant bacterial genera (microbiome)

No statistically significant mediation effect of NLR was observed in the comparison between controls and smokeless tobacco users (Table 13b).

Table 13b: Mediation analysis of the Control vs smokeless tobacco users effect on significant genera via NLR

Effect	Estimate	Confidence Interval (95%)	p-value
ACME (Indirect Effect)	-1.06	(-6.48-1.72)	0.724
ADE (Direct Effect)	5.87	(-0.89 - 18.68)	0.264
Total Effect	4.80	(-0.561 - 14.81)	0.186
Proportion Mediated	-0.22	(-1.69 - 1.48)	NA

Exposure (X): Tobacco group (C vs T), Mediator (M): NLR, Outcome (Y): significant bacterial genera (microbiome)

MaAsLin2 analysis showed trends of association ($P < 0.05$) between bacterial genera and FVC (L), FEV_1 (L), FEV_1/FVC%, NLR, and hs-CRP; however, the q value remained greater than 0.01 (Table 14).

Table 14: Bacterial genera association with lung function parameters- by MaAsLin2 analysis

Clinical variables	Bacterial genera	Coefficient	Standard Error	N.not.0	pval	qval
FVC (L)	Catenibacterium	1.385	0.346	11	0.007	0.704
	Haemophilus_D	-0.064	0.776	15	0.004	0.981
	Haemophilus_A	-1.413	0.935	15	0.018	0.729
	Arcobacter	-1.548	0.491	11	0.02	0.704
	Moraxella	-0.438	1.299	14	0.02	0.937
	Terribacillus	1.496	0.493	13	0.023	0.704
	Empedobacter	0.538	0.188	12	0.029	0.704
	Enterobacter B	0.616	0.244	15	0.045	0.704
	Streptococcus	0.363	0.724	15	0.045	0.911
FEV_1 (L)	Streptococcus	-0.559	0.742	15	0.003	0.858
	Moraxella	-0.63	1.33	14	0.015	0.9127
	Haemophilus_D	1.118	0.796	15	0.021	0.733
	Haemophilus_A	1.983	0.959	15	0.04	0.704
	Lactococcus	-0.764	0.304	8	0.046	0.704
	Pauljensenia	-1.832	0.73	15	0.046	0.704
FEV_1/FVC%	Porphyromonas_A	1.481	0.547	15	0.035	0.704
	BMS3ABIN17	-0.767	0.299	11	0.042	0.704
	Moraxella	0.586	0.928	14	0.001	0.881
	Streptococcus	0.38	0.517	15	0.01	0.863
	Haemophilus_A	-0.071	0.668	15	0.02	0.973
	Haemophilus_D	0.093	0.554	15	0.03	0.965
	Methyloversatilis	-0.817	0.325	11	0.045	0.704

Clinical variables	Bacterial genera	Coefficient	Standard Error	N.not.0	Pval	Qval
NLR%	Haemophilus_D	0.265	0.942	15	0.002	0.945
	OLB10	0.75	0.189	11	0.007	0.704
	Lactococcus_A	0.836	0.214	8	0.008	0.704
	Campylobacter_A	-1.379	0.355	15	0.008	0.704
	Arcobacter	1.093	0.355	11	0.022	0.704
	Prevotella	-1.204	0.394	15	0.022	0.704
	Veillonella_A	-0.972	0.341	15	0.029	0.704
	Clostridium_T	1.202	0.434	13	0.032	0.704
	Streptococcus	-1.051	0.523	15	0.034	0.704
	Pseudomonas_B	1.347	0.507	14	0.038	0.704
	Leptotrichia_A	-1.39	0.536	15	0.041	0.704
	Haemophilus_A	-0.048	0.676	15	0.049	0.983
	Halococcus	1.01	0.421	13	0.053	0.704
	Alcaligenes	1.075	0.45	15	0.04	0.704
	Moraxella	1.889	0.939	15	0.01	0.704

Clinical variables	Bacterial genera	Coefficient	Standard Error	N.not.0	Pval	qval
hsCRP (mg/dl)	Haemophilus_A	0.765	0.925	15	0.004	0.851
	Empedobacter_	-0.781	0.186	12	0.006	0.704
	Catenibacterium	-1.418	0.342	11	0.006	0.704
	Alistipes_A_	-1.357	0.363	9	0.01	0.704
	Terribacillus	-1.821	0.488	13	0.01	0.704
	M3007	-1.724	0.481	9	0.012	0.704
	UBA9973	-1.944	0.545	9	0.012	0.704
	Tangfeifania	-2.881	0.839	12	0.014	0.704
	Enterobacter_B	-0.826	0.241	15	0.014	0.704
	RHKY01	-2.189	0.671	14	0.017	0.704
	Muribaculum	-2.013	0.65	13	0.021	0.704
	Fimenecus	-1.934	0.642	10	0.024	0.704
	Lachnoanaerobaculum	2.542	0.847	15	0.024	0.704
	Accumulibacter	-2.591	0.863	15	0.024	0.704
	Streptococcus	0.888	0.716	15	0.026	0.759
	Magnetospirillum	-2.426	0.847	11	0.029	0.704
	UBA2329	-1.165	0.41	8	0.03	0.704
	Desulfobulbus_A	-1.509	0.542	11	0.032	0.704
	Moraxella	0.034	1.285	15	0.042	0.995
	Haemophilus_D	-0.561	1.289	15	0.08	0.919
	SZUA.272	-2.362	0.853	13	0.032	0.704
	Solibacillus	-2.195	0.793	13	0.033	0.704
	Anaerocolumna	-2.305	0.834	13	0.033	0.704
	Prevotella	-1.489	0.539	15	0.033	0.704
	Lysobacter_A	-1.933	0.711	10	0.035	0.704

	DT.91	-1.941	0.724	11	0.036	0.704
	Rubrivivax	-2.027	0.771	10	0.039	0.704
	WRKR01	-1.853	0.710	8	0.040	0.704
	Johnsonella	3.011	1.159	9	0.041	0.704
	Gemmatimonas	-1.523	0.597	10	0.043	0.704
	OLB7	-1.004	0.397	11	0.045	0.704
	Clostridium_Q	-1.451	0.576	8	0.045	0.704
	Aquabacterium_A	-1.900	0.757	12	0.046	0.704
	Parabacteroides_B	-1.868	0.749	13	0.047	0.704
	Paenibacillus_J	-1.896	0.762	11	0.047	0.704
	Rodentibacter_A	-1.589	0.656	14	0.052	0.704
	Lacunisphaera	-2.156	0.897	11	0.053	0.704
	UBA2475	-1.818	0.762	12	0.054	0.704
	Arenimonas	-1.829	0.768	12	0.055	0.704

Multivariate Association with Linear Models (version 2) -MaAsLin2

Targeted molecular sequencing

Streptococcus pneumoniae, Haemophilus influenzae, and Moraxella catarrhalis were selected because they were consistently detected in microbiome profiling, and previous literature has reported an association between these pathogens and tobacco exposure. Conventional PCR identified a higher prevalence of these pathogens in both smokers and smokeless tobacco users compared with controls. *Streptococcus pneumoniae* was detected in 23 smokers and 26 smokeless tobacco users, compared with 5 controls. *Haemophilus influenzae* was detected in 25 smokers and 28 smokeless tobacco users, but in only 8 controls. *Moraxella catarrhalis* was detected in only 2 smokers (Table 15).

Table 15: Comparison of respiratory pathogen detection across the study group

Bacterial identification (n =30)	Gene	Smoker	Smokeless tobacco user	Control
Streptococcus Pneumoniae	PsaA2	23	26	5
Haemophilus Influenzae	OMP6	25	28	8
Moraxella Catarrhalis	MCATI	2	0	0

No significant differences were observed in stratified analyses comparing the presence and absence of bacteria with pulmonary function and blood parameters (Tables 16a and 16b).

Table 16a: Stratified analysis of bacterial presence or absence in relation to pulmonary function parameters

	S.P (+) (23)	S.P (-) (7)	P.value	H.I (+) (25)	H.I (-) (5)	P.value
Smoker	median (IQR)			median (IQR)		
FVC (L)	3.3 (3.0 -3.5)	3.3 (3.0 -3.4)	0.76	3.3 (3.0-3.5)	3.5 (3.1 -3.5)	0.86
FEV_1 (L)	2.9 (2.5 -3.2)	2.6 (2.4 -2.9)	0.14	2.9 (2.4 -3.2)	2.9 (2.6 -2.9)	0.57
FEV_1/FVC%	79.3 (76.7 -81.9)	80 (78 -82.3)	0.52	79.3 (76.6 -81.9)	82 (79.3 -82.6)	0.3
PEFR (L/m)	451 (401 -519)	419 (401 -478)	0.49	431 (383 -512)	498 (451 -513)	0.24
$FER_{25-75\%}$ (L/s)	2.6 (2.2 -3.0)	2.5 (2.3 -3.3)	0.76	2.6 (2.3 -3.0)	2.8 (2.6 -3.3)	0.37
Smokeless tobacco user	S.P (+) (25)	S.P (-) (n= 5)		H.I (+) (28)	H.I (-) (2)	p.value
FVC (L)	3.3 (2.6-3.0)	2.7 (2.6 -3.0)	0.24	3.0 (2.7 -3.6)	3.9 (3.6 -4.2)	0.24
FEV_1 (L)	3.0 (2.6 - 3.3)	2.5 (2.5 -3.5)	0.61	2.9(2.6-3.4	2.9(2.8 -3.0)	0.96
FEV_1/FVC%	80 (76 -83)	81 (77.4 -85.2)	0.55	80 (77 -84)	74 (72 -70)	0.15
PEFR (L/m)	455 (434 -536)	480 (472 -499)	0.59	463 (434 -514)	531 (508 -55)	0.26
$FER_{25-75\%}$ (L/s)	2.9 (2.1 -3.7)	2.8 (2.7 -3.8)	0.73	2.9 (2.2 -3.7)	2.6(2.1 -3.1)	0.61
Control	S.P (+) (5)	S.P (-) (25)		H.I (+) (8)	H.I (-) (20)	p.value
FVC (L)	3.7 (3.5-4.0)	3.5 (3.2 - 3.8)	0.34	3.2 (3.0-3.4)	3.7 (3.4 -3.8)	0.4
FEV_1 (L)	2.9 (2.7 -3.3)	3.0 (2.7 -3.2)	0.8	2.7 (2.4 -2.8)	3.1 (2.8 -3.2)	0.3
FEV_1/FVC%	77.3 (77.3 -79.7)	80 (79 -82)	0.1	79.8 (78.9 -80)	80.8(77.9-82.2)	0.4
PEFR (L/m)	474 (448 -540)	504 (485 -540)	0.29	500 (483 -511)	519 (484 -553)	0.4
$FER_{25-75\%}$ (L/s)	2.4 (2.1 -3.4)	3.4 (3.0 - 4.0)	0.31	3.6 (2.1 -4.0)	3.4 (2.8 -4.0)	0.9

Mann–Whitney U test

Table 16b: Stratified analysis of bacterial presence or absence in relation to blood parameters

Smoker	S.P (+) (23)	S.P (-) (7)	p.value	H.I (+) (25)	H.I (-) (5)	p.value
	(median, IQR)			Median (IQR)		
WBC (cells/mm^3)	7700 (6550,8750)	7200 (6750,8210)	0.64	7500 (6300,8300)	8200 (7900,8600)	0.23
Neutrophil (%)	54 (47.3,59.8)	57.3 (53.5,60.7)	0.42	54.00 (49.80,60.20)	58.60 (55.00,62.30)	0.3
Lymphocytes (%)	34 (28.6,39.3)	29.4 (26.6,34)	0.43	34.00 (28.30,39.70)	29.20 (25.00,32.50)	0.27
Eosinophil (%)	3.0(2.3,3.7)	2.3(1.8,3.4)	0.4	2.80 (2.00,3.70)	2.00 (1.30,4.30)	0.89
Basophil (%)	1.0 (0.6,1.0)	0.70 (0.60,0.90)	0.6	0.80 (0.60,1.00)	1.00 (0.70,1.00)	0.6
Monocyte (%)	7.2 (3.0,9.4)	8.0 (2.8,9.0)	0.71	7.20 (3.00,9.70)	8.00 (2.90,8.00)	0.74
NLR	2.0 (2.0,2.0)	1.0 (1.0,2.0)	0.12	1.90 (1.50,2.20)	1.30 (0.90,1.40)	0.2
hs-CRP (mg/dl)	2.9 (1.6,3.8)	4.4 (1.6,4.7)	0.64	2.80 (1.60,3.90)	4.40 (2.90,4.50)	0.44
Smokeless tobacco user	S.P (+) (26)	S.P (-) (4)		H.I (+) (28)	H.I (-) (2)	p.value
WBC (cells/mm^3)	7150(5900.79 67.5)	6050 (5050,7425)	0.36	7000(5800,8042)	7000 (6700,7300)	0.97

Neutrophil (%)	51.50 (48.25,56.00)	53.90 (49.35,58.50)	0.67	50.65 (48.00,56.72)	54.60 (53.90,55.30)	0.62
Lymphocytes (%)	32.05 (28.25,36.95)	30.90 (28.10,33.75)	0.78	31.20 (28.00,36.85)	35.50 (34.75,36.25)	0.34
Eosinophil (%)	7.00 (2.90,11.55)	2.55 (1.08,6.55)	0.27	7.00 (2.80,11.70)	2.15 (1.57,2.72)	0.13
Basophil (%)	0.50 (0.20,0.80)	0.35 (0.08,0.70)	0.46	0.40 (0.10,0.80)	4.80 (2.70,6.90)	0.17
Monocyte (%)	7.25 (6.20,8.38)	5.85 (4.95,7.07)	0.2	7.00 (6.00,8.50)	7.20 (6.65,7.75)	1
NLR	1.45 (1.30,1.80)	1.35 (1.20,1.62)	0.44	1.45 (1.30,1.80)	1.65 (1.48,1.82)	0.93
hs-CRP (mg/dl)	3.10 (2.80,3.80)	4.25 (2.88,7.03)	0.39	3.10 (2.85,3.85)	3.00 (2.90,3.10)	0.6
Control	S.P (+) (5)	S.P (-) (25)		H.I (+) (8)	H.I (-) (22)	p.value
WBC (cells/mm^3)	6600 (6500,8400)	8300 (6900,9400)	0.36	8400 (7025,9725)	7785(6750,9325)	0.83
Neutrophil (%)	54.00 (50.00,58.00)	49.00 (45.00,53.60)	0.11	49.00 (43.75,58.25)	50.85 (46.88,53.90)	0.91
Lymphocytes (%)	29.90 (28.00,35.00)	37.60 (31.00,39.90)	0.11	34.50 (29.42,38.30)	36.35 (30.15,39.67)	0.64
Eosinophil (%)	4.50 (2.90,4.98)	3.00 (1.20,5.00)	0.96	3.00 (2.67,4.62)	3.50 (1.20,5.60)	0.96
Basophil (%)	0.40 (0.40,1.00)	1.00 (0.50,1.00)	0.25	1.00 (0.40,1.00)	1.00 (0.43,1.00)	0.71
Monocyte (%)	9.00 (9.00,9.10)	7.30 (6.00,8.00)	0.4	8.50 (6.75,9.03)	7.65 (6.30,8.00)	0.39
NLR	1.30 (1.30,1.50)	1.40 (1.10,1.70)	0.87	1.45 (1.15,1.68)	1.35 (1.20,1.68)	0.94
hs-CRP (mg/dl)	1.00 (1.00,1.10)	0.60 (0.40,1.00)	0.08	1.05 (0.82,1.38)	0.60 (0.40,1.00)	0.08

Mann–Whitney U test.

Multiple linear regression demonstrated that the presence of *Haemophilus influenzae* was independently and negatively associated with FVC. Smoking was independently and negatively associated with FEV_1 and PEFR (Table 17a). Smoking was independently associated with higher neutrophil (%) and elevated hs-CRP levels, whereas smokeless tobacco use was independently associated with hs-CRP levels alone (Table 17b).

Table 17a: Multiple linear regression analysis of pulmonary function parameters in relation to tobacco use and bacterial colonization (adjusted for age and BMI)

PFT Parameters	Smoker		Smokeless tobacco user		S. Pneumoniae (Presence)		H. Influenza (Presence)	
	β	p.value	β	p.value	β	p.value	β	p.value
FVC (L)	-0.01	0.93	-0.23	0.23	0.20	0.21	- 0.37	0.02*
FEV1(L)	-0.25	0.04 *	-0.03	0.82	0.11	0.26	- 0.09	0.40
FEV1/FVC%	-2.21	0.14	-1.58	0.33	-0.09	0.94	-0.71	0.59
PEFR (L/min)	-58.43	0.01 *	- 41.58	0.09	18.66	0.33	-20.60	0.30
$FER_{25-75\%}$ (L/s)	-0.19	0.51	-0.11	0.73	-0.27	0.27	-0.28	0.26

Multiple Linear Regression

Table 17b: Multiple linear regression analysis of blood parameters in relation to tobacco use and bacterial colonization (adjusted for age and BMI)

Blood parameters	Smoker		Smokeless tobacco user		S. Pneumoniae (presence)		H. Influenza (presence)	
	β	p.value	β	p.value	β	p.value	β	p.value
WBC (cells/mm^3)	-883	0.71	886.06	0.73	0.01	0.93	-0.01	0.92
Neutrophil (%)	5.54	0.02 *	3.22	0.22	0.10	0.44	-0.05	0.70
Lymphocytes (%)	-1.46	0.55	-2.29	0.39	-0.07	0.57	0.01	0.94
Eosinophil (%)	-1.25	0.29	2.26	0.08	0.07	0.55	-0.04	0.73
Basophil (%)	-0.35	0.80	-0.11	0.94	-0.01	0.89	-0.19	0.16
Monocyte (%)	-1.77	0.05	-1.20	0.21	0.18	0.19	-.004	0.94
NLR	0.17	0.44	0.04	0.88	0.19	0.16	0.23	0.07
hsCRP (mg/dl)	3.00	0.001 *	3.09	0.001 *	-0.05	0.65	0.11	0.34

Multiple Linear Regression

9. DISCUSSION

The present study compares the effects of smoking and smokeless tobacco use on the airway microbiome, immunological parameters, and pulmonary function. The findings demonstrate that both smoking and smokeless tobacco use are associated with impaired pulmonary function and increased systemic inflammation. Microbiome profiling indicated that tobacco exposure was associated with changes in specific subsets of bacterial genera, with limited enrichment or depletion observed in both smokers and smokeless tobacco users, rather than a shift in microbial community structure. In addition, pathogenic bacterial species such as *Streptococcus pneumoniae* and *Haemophilus influenzae* were more prevalent in both tobacco-exposed groups.

Pulmonary function tests

Impaired pulmonary function was observed in both smokers and smokeless tobacco users. These findings are consistent with studies by Kharel et al. and Herath et al., which demonstrated that cigarette smoke induces chronic airway inflammation characterized by mucus hypersecretion, ciliary dysfunction, and structural thickening of the airway wall. Such alterations promote airway lumen narrowing and increased airflow resistance, leading to declines in pulmonary function. Similarly, the impaired pulmonary function observed in smokeless tobacco users aligns with reports by Shinde et al. and Gupta et al., who demonstrated that smokeless tobacco use induces systemic inflammation and oxidative stress. These processes may promote inflammatory cell infiltration and structural changes in the airways, thereby contributing to compromised pulmonary function.

Blood parameters

Systemic inflammation observed in both smokers and smokeless tobacco users is consistent with previous studies. Malenica et al. and Gumus et al. reported elevated WBC counts, neutrophil levels, and neutrophil-to-lymphocyte ratios in smokers, while Elisia et al. reported elevated CRP and activation of immune cells in smoking-related systemic responses driven by cytokine release. Similarly, Memon et al. reported higher WBC and CRP among smokeless tobacco users, reflecting an enhanced systemic inflammatory response. In agreement with these reports, the present findings confirm that both smoking and smokeless tobacco use are associated with significant systemic inflammatory responses.

Microbiome profiling

Microbiome profiling indicated that tobacco exposure was associated with alterations in specific subsets of bacterial genera, with limited enrichment or depletion observed in both smokers and smokeless tobacco users, rather than a major shift in microbial community structure. This observation is consistent with findings reported by Vladimir, who demonstrated that tobacco smoke exposure predominantly influences individual bacterial taxa rather than the overall airway microbial structure. These selective alterations may reflect hypoxic conditions in smokers' airways, which favour the growth of facultative anaerobic species.

In the control versus smoker comparison, differential abundance analysis showed enrichment of *Leptotrichia*, *Haemophilus*, *Prevotella*, *Veillonella*, *Porphyromonas*, and *Streptococcus* in smokers, along with reduced abundance of *Staphylococcus*, *Acinetobacter*, and *Ligilactobacillus*. These findings are consistent with those reported by Erb-Downward et al., who similarly observed increased abundance of *Streptococcus*, *Prevotella*, *Haemophilus*, *Veillonella*, and *Porphyromonas* in smokers. In the control versus smokeless tobacco user comparison, smokeless tobacco users showed increased abundance of *Escherichia*, *Pseudomonas*, *Porphyromonas*, *Acinetobacter*, and *Streptococcus*, with a concomitant reduction in *Haemophilus*, *Neisseria*, and *Rothia*. Collectively, these results indicate that both smoking and smokeless tobacco use are associated with airway microbiome alterations, despite differences in the mode of consumption. Tobacco exposure may disrupt mucociliary function and weaken innate immune defenses, thereby facilitating microbial colonization, while the resulting inflammatory responses may further exacerbate impairment of pulmonary defense mechanisms.

Associations were observed between inflammatory markers, including total white blood cell count and hs-CRP, and the microbiome; however, after adjustment for tobacco exposure, only the neutrophil-to-lymphocyte ratio (NLR) remained significantly associated with the microbiome. Mediation analysis was performed to examine whether the association between tobacco exposure and alterations in bacterial genera was mediated by NLR. The absence of a significant mediating effect of the neutrophil-to-lymphocyte ratio (NLR) suggests that changes in the airway microbiome are driven predominantly by the direct effects of tobacco exposure rather than by inflammatory mediation. Tobacco constituents such as nicotine, tar, nitrosamines, reactive oxygen species, and heavy metals can directly influence the airway

microenvironment by altering epithelial surfaces, mucus properties, oxygen gradients, and local immune responses. These direct effects are sufficient to modify bacterial abundance and community composition independently of systemic inflammatory markers, thereby explaining the limited mediating role observed for inflammatory indices. MaAsLin2 analysis suggested a trend toward associations between specific bacterial genera and clinical parameters. In contrast, Morrow et al. reported significant positive associations between bacterial genera (Streptococcus, Staphylococcus, Acinetobacter, Moraxella, Serratia, Cupriavidus, and Bacillus) and neutrophil percentages as well as the neutrophil-to-lymphocyte ratio (NLR).

Targeted molecular sequencing

Streptococcus pneumoniae and *Haemophilus influenzae* were more abundant in both smokers and smokeless tobacco users compared with controls. This finding is consistent with Moore et al., who reported increased *Haemophilus influenzae* colonization in smokers. Tobacco-induced impairment of mucociliary clearance and innate immune defense likely facilitates pathogen persistence and chronic colonization. No significant differences were observed in stratified analyses comparing the presence and absence of bacteria with clinical variables. However, multiple linear regression demonstrated that smoking was significantly associated with reduced FEV_1 and PEFR, along with increased neutrophil (%) and hs-CRP levels. In addition, *Haemophilus influenzae* colonization showed an independent negative association with FVC. Whereas smokeless tobacco use was independently associated with hs-CRP levels alone. These findings are consistent with those reported by Ghosh et al., who observed that increased abundances of *Streptococcus pneumoniae* and *Haemophilus influenzae* in smokers were negatively correlated with FEV_1%, FVC%, and FEV_1/FVC%. Collectively, these results suggest that airway colonization by pathogenic bacteria may contribute to lung function decline through sustained airway inflammation, mucin adherence, immune evasion, and persistent infection.

10. LIMITATIONS

The cross-sectional design limits causal inference.

The small metagenomic sample size restricts the strength of clinical associations.

Possible oropharyngeal contamination of sputum samples may affect microbiome profiles.

11. CONCLUSION

In conclusion, both smoking and smokeless tobacco use are associated with impaired pulmonary function and increased systemic inflammation. Smokers exhibit more extensive functional deficits accompanied by predominantly neutrophil-mediated inflammatory responses, whereas smokeless tobacco users demonstrate inflammation characterized by eosinophil predominance. Although the respiratory microbiome shows group-specific shifts in selected bacterial genera, these alterations do not independently influence pulmonary or immunological parameters, with the observed associations largely attributable to direct tobacco exposure. Furthermore, tobacco use is associated with increased detection of key respiratory pathogens. Notably, *Haemophilus influenzae* colonization showed an independent negative association with forced vital capacity (FVC), indicating early microbial alterations within the airway environment. However, the interpretation of these findings is limited by the relatively small sample size.

12. SUMMARY

All participants underwent pulmonary function testing, complete blood counts, hs-CRP estimation, 16S rRNA gene amplicon sequencing, and targeted molecular sequencing. Both tobacco-using groups showed reduced pulmonary function and elevated systemic inflammatory markers. The microbiome profile revealed only small subsets of bacterial genera that were enriched or depleted in tobacco users, rather than a major shift in the overall community structure, with tobacco exposure directly influencing these specific subsets. Based on these 16s rRNA gene amplicon sequencing findings, targeted molecular sequencing was performed and showed increased detection of Streptococcus pneumoniae and Haemophilus influenzae, with H. influenzae demonstrating a negative association with FVC. The study suggests that irrespective of mode of consumption, tobacco use impairs lung function, increases systemic inflammation, alters specific bacterial genera, and promotes colonization by pathogenic bacteria.

13. FUTURE RECOMMENDATIONS

Include female participants to enhance the generalizability of the findings.

A larger sample size would improve understanding of the associations between the microbiome and clinical variables

Apply shotgun metagenomic sequencing to enable species- and strain-level resolution beyond 16S rRNA analysis and to provide insights into airway microbiome alterations.

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15. RESEARCH PUBLICATIONS FROM THE STUDY

1. Sathiyaseelan M, Srinivasan K, Raj JB, Ranganadin P, Subramanian B. Potential health impacts and lung microbiome changes among smoking and smokeless tobacco use: a technical scan. *Eurasian J Pulmonol*. 2021;23:77–82.

2. Early indicators of alterations in hematological and inflammatory biomarkers among smoking and smokeless tobacco users: a comparative analysis. *Int J Environ Sci*. 2025;11(6):90–95

3. Sathiyaseelan M, Jothimani P, Arasan M, et al. Nicotine dependence, lung function decline, and respiratory pathogen colonization in tobacco smokers: a molecular and functional insight study. *J Pure Appl Microbiol*. 2025;19(4):2912–2922.

16. PAPER & POSTER PRESENTATIONS

1. Oral Presentation: Potential health impacts and lung microbiome changes among smokeless and smoking tobacco users, presented at SBV Research Days 2020, organized by the Deanery of Research, Sri Balaji Vidyapeeth, Puducherry.

2. Oral Presentation: Nicotine dependence, lung function, and respiratory pathogen colonization in tobacco smokers: a molecular and functional insight study was orally presented at APTCON 2025 (YAKKAI 360°), the 17th Annual Conference of the Association of Physiologists of Tamil Nadu, conducted by AIIMS Madurai

17. Conferences/CME/Workshops Attended

1. FDP on *"Guidelines for Applying Extramural Research Grants"*, held on 19–20 November 2019, organized by the Deanery of Research, SBV.

2. Attended a two-day webinar workshop titled *"Thesis Writing Workshop"* for Ph.D. scholars, organized by the Deanery of Research, Sri Balaji Vidyapeeth (SBV), Puducherry, held on 16–17 October 2020.

3. Participated in a two-day National Continuing Medical Education (CME) programme on "Current Trends in Epidemiology and Biostatistics", organized by the Mahatma Gandhi Medical Advanced Research Institute in collaboration with ICMR–National Institute of Epidemiology (NIE), Chennai, at Sri Balaji Vidyapeeth (SBV), Puducherry, held on 10th and 11th July 2024.

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Review Article

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Potential health impacts and lung microbiome changes among smoking and smokeless tobacco use : A technical scan

Manikandan Sathiyaseelan, Krishnan Srinivasan, Jeneth Berlin Raj, Pajanivel Ranganadin[1], Balanehru Subramanian[2]

ORCID:
Manikandan Sathiyaseelan: https://orcid.org/0000-0001-6695-3814
Krishnan Srinivasan: https://orcid.org/0000-0002-0673-1643
Jeneth Berlin Raj: https://orcid.org/0000-0001-5412-4824
Pajanivel Ranganadin: https://orcid.org/0000-0002-2608-1633
Balanehru Subramanian: https://orcid.org/0000-0002-7010-1486

Abstract:

Globally, 1.3 billion peoples use tobacco products daily, most of them from low- and middle-income countries. Every year, 8 million of the population died because of the usage of tobacco, as per the WHO report. Tobacco-related diseases, likely lung cancer, emphysema chronic obstructive pulmonary disease, and chronic respiratory diseases, are more common. Nicotine is a highly addictive chemical found in the tobacco plant and also in all other tobacco products. All tobacco products contain nicotine that causes the release of dopamine in the pleasure and motivation areas of the brain. Both smoking and smokeless tobacco products contain many harmful chemicals that affect the lung mucociliary clearance and also inactivate the immune system and lead to increases in the risk of infection and the development of deleterious bacterial growth in the lungs. In the lung microbiome, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Pseudomonas aeruginosa* bacterial species were found to a higher level in smoking tobacco users as that may increase morbidity and mortality. The lung microbiome changes taking place in the lung due to smokeless tobacco usage are less documented so far. In future research, microbiome studies help to understand disease pathogenesis.

Keywords:

Bacterial growth, inflammatory changes, tobacco users

Departments of Physiology and [1]Pulmonary Medicine, Mahatma Gandhi Medical College and Research Institute, Sri Balaji Vidyapeeth, [2]Director, Central Inter-Disciplinary Research Facility, Sri Balaji Vidyapeeth, Puducherry, India

Address for correspondence:
Dr. Krishnan Srinivasan, Department of Physiology, Mahatma Gandhi Medical College and Research Institute, Puducherry, India.
E-mail: drkrish10@gmail.com

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Introduction

Globally, every year, 8 million of the population died because of the usage of tobacco, as per the WHO report. Around 7 million populations died because of the direct use of tobacco and nearly 1 million population died indirectly by secondhand smoke.[1] In India, tobacco kills more than 1 million people every year.[2] India is the second-largest population of tobacco users in the world. Different forms of tobacco products are available in India. Tobaccos in smoking and smokeless products are injurious to health. Tobacco users are associated with noncommunicable diseases like ischemic heart diseases, cancers, chronic respiratory diseases like chronic obstructive pulmonary disease (COPD), emphysema, and bronchitis which are leading causes of death globally.[3] Tobacco smoke contains 7000 chemicals, in

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Early Indicators Of Alterations In Hematological And Inflammatory Biomarkers Among Smoking And Smokeless Tobacco Users - Comparative Analysis

Manikandan Sathiyaseelan[1], Vickneshwaran Vinayagam[2], Pradeep Jothimani[3], Pajanivel Ranganadin[4], Balanehru Subramanian[5], Richa Gupta[6], Nikhilesh Singh[7]

[1]PhD scholar &Tutor, Department of Physiology, Mahatma Gandhi Medical College and Research Institute, Sri Balaji Vidyapeeth (Deemed to be University), Puducherry, India,
manikandan2303@gmail.com

[2]Assistant Professor, Department of Biochemistry, Mahatma Gandhi Medical College and Research Institute, Sri Balaji Vidyapeeth (Deemed to be University), Puducherry, India

[3]Associate Professor, Mahatma Gandhi Medical Advanced Research Institute, Sri Balaji Vidyapeeth (Deemed-to-be-University) Puducherry

[4]Professor & Head, Department of Pulmonary Medicine, Mahatma Gandhi Medical College and Research Institute, Sri Balaji Vidyapeeth (Deemed to be University), Puducherry, India

[5]Principal, School of Biomedical Sciences, Sri Balaji Vidyapeeth (Deemed to be University), Pillaiyarkuppam, Puducherry, India

[6]Professor, Department of Physiology, Mahatma Gandhi Medical College and Research Institute Sri Balaji Vidyapeeth (Deemed to be University), Pillaiyarkuppam, Puducherry, India.

[7]***Corresponding Author**
Professor, Department of Physiology, Mahatma Gandhi Medical College and Research Institute Sri Balaji Vidyapeeth (Deemed to be University), Pillaiyarkuppam, Puducherry, India.

Abstract

Background: *In India, 1 million tobacco-related deaths occur every year. Consumption of smoking and smokeless tobacco products was a more common practice. This study aimed to assess and compare hematological and inflammatory markers among smoking and smokeless tobacco users.*

Material and methods: *Ninety participants were recruited and divided into three groups: 30 smokers, 30 smokeless tobacco users, and age- and BMI-matched 30 healthy controls. The levels of hsCRP and complete blood count (CBC) were measured.*

Results: *Significant differences were observed in mean corpuscular volume (MCV) and neutrophil levels in both tobacco user groups compared to controls. Eosinophils were reduced in smokers but elevated in smokeless tobacco users, while basophils and monocytes were decreased in both groups. hsCRP levels were markedly elevated in both smokers and smokeless tobacco users, indicating systemic inflammation. The neutrophil-to-lymphocyte ratio (NLR) was significantly increased among smokers, suggesting a stronger systemic impact of smoking compared to smokeless tobacco use.*

Conclusion: *These findings emphasize the need for early monitoring of hematological and inflammatory biomarkers among tobacco users. Significant changes in MCV, neutrophils, eosinophils, basophils, monocytes, and elevated hsCRP levels and NLR suggest systemic inflammation and altered immune responses in tobacco users. While some hematological parameters remained unchanged, elevated inflammatory markers such as hsCRP and NLR in smokers indicate a stronger systemic impact of tobacco use. This study helps to guide targeted public health interventions and preventive strategies.*

Keywords: *Smokers, Smokeless tobacco users, High sensitive C-reactive protein, Neutrophil-Lymphocytes ratio. Systemic inflammation, hematological changes*

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RESEARCH ARTICLE OPEN ACCESS

Nicotine Dependence, Lung Function Decline, and Respiratory Pathogen Colonization in Tobacco Smokers: A Molecular and Functional Insight Study

Manikandan Sathiyaseelan[1], Pradeep Jothimani[2], Mashila Arasan[2], Pajanivel Ranganadin[3], Balanehru Subramanian[4], Richa Gupta[1] and Nikhilesh Singh[1]*

[1]Department of Physiology, Mahatma Gandhi Medical College and Research Institute, Sri Balaji Vidyapeeth (Deemed-to-be-University), Pillaiyarkuppam, Puducherry, India.
[2]Department of Microbiology, Mahatma Gandhi Medical Advanced Research Institute, Sri Balaji Vidyapeeth (Deemed-to-be-University), Puducherry, India.
[3]Department of Pulmonary Medicine, Mahatma Gandhi Medical College and Research Institute, Sri Balaji Vidyapeeth (Deemed-to-be-University), Pillaiyarkuppam, Puducherry, India.
[4]School of Biomedical Sciences, Sri Balaji Vidyapeeth (Deemed-to-be-University), Pillaiyarkuppam, Puducherry, India.

***Correspondence:** nikhileshsingh111@gmail.com

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